Detection of specific antibodies in dogs infected with Angiostrongylus vasorum

Schucan, A ; Schnyder, M ; Tanner, I ; Barutzki, D ; Traversa, D ; Deplazes, P

Abstract: Canine angiostrongylosis, caused by the nematode Angiostrongylus vasorum, is an emerging cardiopulmonary disease in Europe which can be fatal if left untreated. We determined the diagnostic value of the specific detection of antibodies against A. vasorum adult somatic antigen, adult excretory/secretory (E/S) antigen and first stage larvae (L1) somatic antigen in ELISAs. Also, A. vasorum adult somatic antigen purified by monoclonal antibodies (mAb) was evaluated in a sandwich-ELISA. Among the crude antigens, the best sensitivities when testing 21 naturally infected dogs were obtained using adult E/S and somatic antigen (85.7% and 76.2%, respectively), which were comparable with the results of the sandwich-ELISA based on mAb-purified antigens (81%). The ELISA performed with L1 antigen had the lowest sensitivity (42.9%). In experimentally inoculated dogs, the sensitivities ranged from 97.7% to 100% with all test settings. The specificity was 98.8% (92.5-99.9%, 95% CI) with all ELISAs using sera of 82 randomly selected dogs. Cross-reactions using adult somatic, adult E/S and L1 somatic antigen were observed in sera of dogs infected with Crenosoma vulpis, Dirofilaria immitis, Dirofilaria repens, and Eucoleus aerophilus. In contrast, using the mAb-purified antigens, the cross-reactions were minimal. Depending on the antigens used, specific antibodies were detected starting between 13 and 21 days post experimental inoculation (dpi), and at latest between 35 and 48 dpi, thus before or around the onset of patency. The serological follow-up of four A. vasorum-infected dogs after anthelmintic treatment at 88 dpi showed a decrease of antibody levels after drug administration, and the animals became seronegative 2-9 weeks later. Two untreated dogs remained seropositive. In four dogs treated 4 dpi, virtually no antibody-reaction was detectable, with the exception of the ELISA performed with L1 antigen. The early detection of specific antibodies against A. vasorum by ELISA represents a valid alternative for a reliable diagnosis and for follow-up investigations after anthelmintic treatment.

DOI: https://doi.org/10.1016/j.vetpar.2011.09.040

Detection of specific antibodies in dogs infected with *Angiostrongylus vasorum*

A. Schucan [a], M. Schnyder [a], I. Tanner [a], D. Barutzki [b], D. Traversa [c], P. Deplazes [a,*]

[a] *Institute of Parasitology, Vetsuisse Faculty, University of Zurich, Winterthurerstr. 266a, CH-8057 Zurich, Switzerland*

[b] *Veterinary Laboratory Freiburg, 79120 Freiburg i.Br., Germany*

[c] *Department of Comparative Biomedical Sciences, University of Teramo, Piazza Aldo Moro 45, Teramo 64100, Italy*

* *Corresponding author. Tel. +41 44 635 85 02; fax: +41 44 635 89 07 E-mail address: deplazesp@access.uzh.ch*

Abstract

Canine angiostrongylosis, caused by the nematode *Angiostrongylus vasorum*, is an emerging cardiopulmonary disease in Europe which can be fatal if left untreated. We determined the diagnostic value of the specific detection of antibodies against *A. vasorum* adult somatic antigen, adult excretory/secretory (E/S) antigen and first stage larvae (L1) somatic antigen in ELISAs. Also, *A. vasorum* adult somatic antigen purified by monoclonal antibodies (mAb) was evaluated in a sandwich-ELISA. Among the crude antigens, the best sensitivities when testing 21 naturally infected dogs were obtained using adult E/S and somatic antigen (85.7% and 76.2%, respectively), which were comparable with the results of the sandwich-ELISA based on mAb-purified antigens (81%). The ELISA performed with L1 antigen had the lowest sensitivity (42.9%). In experimentally inoculated dogs, the sensitivities ranged from 97.7 to 100% with all test settings. The specificity was 98.8% (92.5-99.9%, 95% CI) with all ELISAs

using sera of 82 randomly selected dogs. Cross-reactions using adult somatic, adult E/S and L1 somatic antigen were observed in sera of dogs infected with *Crenosoma vulpis*, *Dirofilaria immitis*, *Dirofilaria repens*, and *Eucoleus aerophilus*. In contrast, using the mAb-purified antigens, the cross-reactions were minimal. Depending on the antigens used, specific antibodies were detected starting between 13 and 21 days post experimental inoculation (dpi), and at latest between 35 and 48 dpi, thus before or around the onset of patency. The serological follow-up of four *A. vasorum*-infected dogs after anthelmintic treatment at 88 dpi showed a decrease of antibody levels after drug administration, and the animals became seronegative 2-9 weeks later. Two untreated dogs remained seropositive. In four dogs treated 4 dpi, virtually no antibody-reaction was detectable, with the exception of the ELISA performed with L1 antigen. The early detection of specific antibodies against *A. vasorum* by ELISA represents a valid alternative for a reliable diagnosis and for follow-up investigations after anthelmintic treatment.

Key words: *Angiostrongylus vasorum*, dog, diagnosis, ELISA, serology.

1. Introduction

The currently most employed diagnostic method for the detection of *Angiostrongylus vasorum* infection, which can cause a potentially lethal disease in dogs, is based on the isolation of first stage larvae (L1) in faeces applying larval migration techniques such as the Baermann-Wetzel technique (Eckert et al., 2008). Repeated testing of faecal samples is suggested to increase the diagnostic sensitivity because of intermittent larval excretion (Oliveira-Junior et al., 2006). However, in cases of low worm burdens and when analysing posted faecal samples that arrive with delay at the laboratory and therefore contain inactive or dead larvae, sensitivity is

reduced and morphological differentiation from other lung worm larvae such as *Crenosoma vulpis* and *Filaroides* spp. is challenging (Eckert et al., 2008; McGarry and Morgan, 2009). Also, larvae should be differentiated from those of *Strongyloides* and/or hookworms that may be present in delayed samples, and from free-living or plant parasitic nematodes that may occur in samples collected from the ground.

FLOTAC, a technique based on the counting of larvae in chambers after spinning faecal samples onto a surface, is suggested to improve the diagnosis of *A. vasorum*, especially when larval migration techniques cannot be used (Schnyder et al., 2010b). However, this method is laborious and thus not applicable under some circumstances, e.g. for extended epidemiological investigations. With both, FLOTAC and Baermann-Wetzel technique, prepatent infections are not detected. As alternatives, novel diagnostic tools have been developed. A sandwich-ELISA using rabbit serum directed against whole adult worms was investigated for the detection of circulating *A. vasorum* antigen (Verzberger-Epshtein et al., 2008) and an ELISA using monoclonal and polyclonal antibodies has very recently been described (Schnyder et al., 2011). Furthermore, real-time PCR with DNA from blood or faeces has been evaluated with samples spiked with L1 (Jefferies et al., 2009), and a 'sieve-PCR' developed to facilitate the analysis of larger faecal volumes may be used as a non-invasive tool for wildlife surveillance and for confirmative diagnosis in definitive hosts (Al-Sabi et al., 2010). In particular, molecular techniques are recommended for prevalence studies in intermediate mollusc hosts (Ferdushy et al., 2009). Few studies have described immunological reactions in dogs infected with *A. vasorum* which could potentially be exploited for the serological detection of such infections. In experimentally infected dogs, specific antibodies against *A. vasorum* adult somatic antigen were detected in ELISA starting 14 days post inoculation (dpi) (Cury et al., 1996). In this study, the pre-absorption of sera with extracts of *Toxocara canis* increased the specificity of antibody detection. However, cross-

reactivity was still observed and was attributed to the possible presence of other helminths. A recently described ELISA, again using somatic antigen of adult *A. vasorum* parasites collected from red foxes, confirmed older results and demonstrated cross-reactivity with sera of dogs infected with *C. vulpis, T. canis* and *D. immitis* (Jefferies et al., 2011a). Western blot analyses of adult somatic antigen with sera of experimentally infected dogs revealed immunogenic antigens of different molecular weight (Cury et al., 2002). Analysing adult and L1 extracts, stage specific antigens of *A. vasorum* were identified and no cross-reactions were detected with sera from dogs naturally parasitized by intestinal helminths (De Oliveira Vasconcelos et al., 2008). Jefferies et al. (2011a) identified a dominant 30 kD band when immunoblotting canine serum samples confirmed positive for *A. vasorum* against somatic antigen of adult *A. vasorum* but slight cross reactions with serum samples from dogs infected with *C. vulpis, T. canis* and *D. immitis* were observed in the same range.

No immunological diagnostics for the detection of *A. vasorum* infections are currently commercially available and no purified or recombinant antigens have been produced. The aim of this study was to investigate the diagnostic values of native crude and purified antigens for the specific detection of antibodies directed against *A. vasorum* in naturally and experimentally infected dogs and to follow the dynamic of the specific antibody reactions in experimentally infected dogs.

2. Materials and methods

2.1. Dog sera

Sera from dogs experimentally infected with *A. vasorum* were derived from previously reported studies. In particular, sera from dogs treated with a spot-on solution containing imidacloprid 10 mg/kg and moxidectin 2.5 mg/kg body weight (Advocate®, Bayer Animal

97 Health) at 4 (n = 5) or 32 (n = 5) days post inoculation (dpi) during prepatency (Schnyder et
98 al., 2009), sera from 4 dogs which were treated at 88 dpi but continued to excrete L3 until 100
99 – 108 dpi (Schnyder et al., 2010a) and sera from two untreated dogs were available.
100 Additionally, sera from 21 naturally infected dogs showing clinical signs compatible with *A.*
101 *vasorum* infection and confirmed for larval excretion by the Baermann technique were
102 collected before treatment was initiated. Thirty sera from healthy blood donor dogs (which are
103 intensively subjected to clinical and laboratory analyses) not originating from a known *A.*
104 *vasorum* endemic area in Switzerland were used to determine the cut-off value, 27 of them
105 showing a positive antibody reaction against *T. canis* E/S antigen (Fahrion et al., 2008). Dogs
106 were not analysed by Baermann-Wetzel technique for exclusion of larval excretion.
107 Specificity was determined with randomly selected sera of 82 dogs presented at the Clinic for
108 Small Animals, and tested at the Clinical Laboratory of the Vetsuisse Faculty of the
109 University of Zurich for various reasons excluding suspected parasitological infections, and
110 with sera collected over three years in Germany from 52 dogs showing one or more clinical
111 signs compatible with angiostrongylosis but without larval shedding as investigated with the
112 Baermann technique (Barutzki and Schaper, 2009). Potential cross-reactions were tested with
113 sera from dogs experimentally infected with *Ancylostoma caninum* (n=4, from the Institute of
114 Parasitology, University of Veterinary Medicine, Hannover), from dogs naturally infected
115 with *Crenosoma vulpis* (n=9) diagnosed by the presence of L1 in faeces (Barutzki and
116 Schaper, 2009), from dogs infected with *Dirofilaria immitis* (n=20), diagnosed by the
117 presence of circulating antigen (DiroCHEK® Synbiotics, San Diego, USA) and/or
118 microfilariae (which were characterized with the acid phosphatase stain), from Italian dogs
119 tested positive for the presence of *Dirofilaria repens* microfilariae by Knott-test and
120 confirmed by PCR (n=5, Traversa et al., 2010) or *Eucoleus aerophilus* (syn. *Capillaria*
121 *aerophila)* eggs detected by coproscopy after flotation and confirmed by PCR-coupled

sequencing (n=6, Traversa et al., 2011), and from Swiss dogs with alveolar liver echinococcosis (n= 8) diagnosed as previously described (Staebler et al., 2006).

2.2. Antigens

A. vasorum adult E/S antigen was obtained as previously described (Schnyder et al., 2011) from female, adult parasites which were isolated from experimentally infected dogs (Schnyder et al., 2009). Adult somatic antigen was produced from *A. vasorum* specimens isolated at necropsy from foxes and dogs and stored at –20° C in phosphate buffered saline (PBS). In a second step, worms were homogenised in a Mixer Mill MM 300 device (Retsch GmbH, Haan, Germany) at 30 Hz for 1 min using a steel bead (diameter 3 mm, Schieritz & Hauenstein AG, Arlesheim, Switzerland). After three freezing/thawing steps using liquid nitrogen and a water-bath, the parasite material was ultrasonicated (Sonifier II W-250, Branson, Danbury, Connecticut, USA) and centrifuged at 14'000 g for 20 min at 4° C. The supernatant containing the soluble somatic antigens was stored at -20° C until further use. Larval somatic antigen was obtained after isolation of *A. vasorum* L1 from faeces of experimentally infected dogs (Schnyder et al., 2009; Schnyder et al., 2010a) by the Baermann-Wetzel technique. Larval suspension was washed four times with PBS containing 200 IU penicillin/ml, 200 µg streptomycin/ml and 0.25 µg amphotericin B/ml (GIBCO) by centrifugation (250 g, for 5 min at +24° C). Afterwards, larvae were collected after migration through a 20 µm mesh sieve at room temperature over night. After a further centrifugation step, the sediment containing the larvae was treated as described above for the production of the *A. vasorum* adult somatic antigen. Protein concentrations were assessed by a Bio-Rad protein assay.

146 *2.3. Monoclonal antibodies*

147 Production of monoclonal antibodies (mAb) against *A. vasorum* adult E/S antigen was based
148 on a previously modified protocol (de StGroth and Scheidegger, 1980), already described in
149 detail by Schnyder et al. (2011). Antibody production of the cells was checked by an ELISA
150 using *A. vasorum* adult E/S antigen. Supernatants positive in the primary screening were
151 subsequently tested for specificity in ELISA with *D. immitis* adult E/S antigen (Deplazes et
152 al., 1995). Selected clones were subcloned twice to assure that the antibody production was
153 monoclonal. Clones positive for *A. vasorum* adult E/S and negative for *D. immitis* adult E/S
154 antigen were cultivated and supernatants concentrated as described (Schnyder et al., 2011).
155 Isotypes were determined using a monoclonal antibody isotyping kit (Ap/PNPP, Pierce,
156 Rockford, Illinois, USA).

157

158 *2.4. ELISA*

159 ELISAs were performed with the optimal antigen, antibody and conjugate concentrations
160 according to the titration results. All test runs included a background control, a conjugate
161 control and three control sera from a naturally infected dog (diluted 1:200), an experimentally
162 infected dog (diluted 1:20,000) and an uninfected blood donor dog (diluted 1:200).

163 *2.4.1. ELISA using crude* A. vasorum *antigens*

164 Micro-ELISA plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated (100 µl/well) either
165 with *A. vasorum* adult E/S antigen (5 µg/ml), *A. vasorum* adult somatic antigen (5 µg/ml) or
166 *A. vasorum* L1 somatic antigen (10 µg/ml) diluted in 0.1 M carbonate/bicarbonate coating
167 buffer (pH 9.6) and stored at 4° C in a humid chamber over night. Plates were washed five
168 times with physiological NaCl/03% Tween-20 (NaCl-T) and saturated for 30 min at 37° C

with PBS (pH 7.2) containing 0.02% NaN_3, 0.05% bovine haemoglobin (Fluka) and 0.2% Tween-20 (PBS-T). Sera were used in a standard dilution of 1:200 in PBS-T additionally containing 10% foetal calf serum (fcs), and 100 µl diluted sera/well were incubated at 37° C in a humid chamber for one hour. After washing five times with NaCl-T, the incubation and washing steps were repeated with the conjugate. For this purpose, specific affinity-purified, alkaline phosphatase conjugated goat anti-dog IgG (γ) antibodies (Kirkegaard and Perry Lab. Inc., Gaithersburg, MD, USA) were used at a dilution of 1:250 in PBS-T (100 µl per well). After the repetition of the washing steps, 100 µl/well of a 1 mg/ml solution of 4-nitrophenyl phosphate (Sigma-Aldrich, Buchs, Switzerland) in 0.05 M carbonate/bicarbonate buffer (pH 9.8) containing 1 mM MgCl were added and incubated for 10 min at 37° C. Absorbance values were read at 405 nm in a Multiscan RC ELISA reader (Thermo Labsystems, Helsinki, Finland). The same methodology was performed for the selection of mAbs: the plates were coated (100 µl/well) with *A. vasorum* adult E/S (5 µg/ml) or *D. immitis* adult E/S antigen (5 µg/ml). Supernatants of the cell cultures were diluted 1:2 in PBS-T and specific affinity purified goat anti-mouse IgG (heavy plus light chains) antibodies conjugated to alkaline phosphatase (Sigma) were used at a concentration of 1:5'000 in PBS-T.

2.4.2. ELISA using mAb-based purified A. vasorum *adult somatic antigen*

A sandwich-ELISA coating with mAbs was used to select potentially diagnostic antigens isolated from the *A. vasorum* adult somatic antigen. The mAbs were used at concentrations of 1 µg/ml or 5 µg/ml, depending on the results of titration experiments. Adult somatic antigen of *A. vasorum* was diluted at 10 µg/ml in PBS-T. Dog sera, conjugate and substrate were used as previously described. In order to further select mAbs with potential for diagnostic use, this sandwich-ELISA was performed with 12 selected test sera (from 3 infected and 9 uninfected

192 animals). Further samples were tested if a promising discrimination between positive and
193 negative sera was observed.

194

195 *2.5. Statistical analysis, animal permissions*

196 Excel 2007 for Windows (Microsoft Corporation, Redmond, USA) was used to calculate
197 means and standard deviations (SD). Sensitivities were calculated dividing the number of
198 seropositive animals by the total number of infected animals of each sample group, while
199 specificities were calculated dividing the number of seronegative animals by the total number
200 of uninfected animals tested. Exact binomial 95% confidence intervals (CI) for means of
201 binomial variables were calculated with unweighted data according to the method of Clopper
202 and Pearson (1934).

203 Experiments with dogs, mice and rabbits were conducted according to the Swiss guidelines
204 for animal experimentation and approved by the Cantonal Veterinary Office of Zurich prior
205 to study start. They were carried out with facility-born animals at the experimental units of
206 the Vetsuisse Faculty in Zurich (permission numbers 25/2006, 26/2007, 13/2008, 185/2008).

207

208 **3. Results**

209 *3.1. Sensitivity and specificity*

210 Sera of dogs naturally and experimentally infected with *A. vasorum* or other parasites as well
211 as sera from healthy dogs and dogs with circulatory symptoms (Barutzki and Schaper, 2009)
212 were used to determine the diagnostic values of the ELISAs (Tables 1 and 2).

213 **Crude antigens:** For natural, patent *A. vasorum* infections, high and comparable sensitivities
214 of 76.2-100% were obtained with the adult E/S and the somatic antigen. Using L1 somatic

antigen, the sensitivity of detection of the naturally infected dogs was only 43%. However, no change in sensitivity of this antigen was observed when samples were tested from experimentally infected animals (Table 1). The specificity was relatively high (98.85%) with all antigens when determined by testing 82 randomly selected sera of Swiss dogs presented for reasons other than suspected parasitological infections. The specificity was 83-87% (Table 1) when samples were evaluated from a group of dogs from Germany with cardiopulmonary signs. These dogs were negative for *A. vasorum* larvae in pooled faecal samples collected over 3 days and tested with the Baermann-Wetzel technique.

Cross-reactions were tested with dogs with proven parasitic infections (Table 2). No positive ELISA results were obtained with sera of dogs with patent *A. caninum* infections and dogs with alveolar echinococcosis in all tests. Cross-reactions occurred in 22.2% of dogs naturally infected with *C. vulpis* with all three crude antigens tested. Sera of dogs with *D. immitis* infection were cross-reacting using the E/S antigen (5%), the L1 antigen (10%) and the adult somatic antigen (20%). Sera from dogs naturally infected with *D. repens* showed more pronounced cross-reactions (40% using the E/S antigen, 100% using the adult somatic antigen and 80% using the L1 antigen). Seventeen percent of the sera from dogs tested positive for the presence of *E. aerophilus* exceeded the cut-off-value using the E/S antigen, 50% using the adult somatic antigen and 17% using the L1 antigen.

Purified antigens: Two mAbs (mAb Av 70/1 and mAb 5/5, both of IgG1 isotype but with differing banding patterns in WB-analyses, results not shown) were selected for the ELISAs with purified somatic antigens. The sensitivity of both these tests was comparable with the ELISAs performed with somatic or E/S adult antigens. However, the specificities with the sera from dogs with clinical signs were higher by trend, but not significantly different (Table

1) and cross-reactions were limited, especially concerning sera of dogs infected with *D. immitis, D. repens* and *E. aerophilus* (Table 2).

3.2. Detection of specific antibodies during experimental A. vasorum *infections*

Experimentally infected dogs seroconverted between 13 and 21 dpi, depending on the antigens used (Fig. 1). The earliest specific antibody detection was at day 13 (5 of the 6 dogs were positive) with the L1 antigen. Specific antibody reactions against the E/S adult antigen were found at 20 dpi (Fig. 1: 1 of 6 animals; Fig. 2: 3 of 5 dogs). The somatic adult antigen revealed first specific reactions at 34 dpi (2 of 6 animals, Fig. 1) and at 21 dpi (1 of 5 animals).

In both ELISAs with purified antigens, a sharp increase of specific antibodies was depicted at 42 dpi in all dogs. Larval excretion started between 47 and 55 dpi (data not shown). Specific antibody levels increased in all ELISAs until 42-50 dpi, thereafter, persistent or slightly declining positive ELISA values were observed during the experimental infection and before treatment (Fig. 1).

Declining antibody levels below the cut-off-value with all adult antigens were observed in four dogs after treatment (88 dpi, Fig. 1). Antibody decrease below the threshold required 2 - 9 weeks, depending on the intensity of antibody reactions before the treatment, and the ELISA adopted. In contrast, specific antibody levels persisted in 2 dogs without treatment (Fig.1). Five dogs already treated at 4 dpi showed no specific reactions against both adult antigens, with one exception (reactions just above the cut-off-value). However, using the L1 somatic antigen, a positive peak around 21-35 dpi in 3 of these 5 dogs was observed (Fig. 2, right side). Interestingly, no antibody increase with the same sera was observed against the mAb-purified antigens (Fig. 2). In contrast, dogs treated at 32 dpi (still in the pre-patent

period) showed positive antibody reactions until the end of the experiment at 55 dpi (Fig. 2, left side) in almost all cases.

4. Discussion

Comparing crude antigens of *A. vasorum* (adult E/S antigen, adult somatic antigen and L1 somatic antigen), a high sensitivity in naturally infected dogs was obtained using both, adult E/S and somatic antigen. In a previous experimental study using adult somatic antigens (Cury et al., 1996), no indications concerning sensitivity were given. In the same study, anti - *A. vasorum* antibodies were detected starting from 14 dpi, however, some dogs remained antibody-negative or became positive only 140 dpi, and potential cross-reactions were not excluded. De Oliveira Vasconcelos et al. (2008) also detected specific antibodies early after inoculation (15 dpi) and evaluated few, taxonomically not closely related helminths (*Dipylidium caninum, Ancylostoma caninum, T. canis*) for cross-reactions. Also Jefferies et al. (2011a) tested potential cross-reactivity with *C. vulpis, T. canis* and *D. immitis* and accordingly performed a cut-off adaptation in order to discriminate *A. vasorum*-positive sera from sera of dogs infected with the cited helminths. Further cross-reactivity against *D. repens* or *E. aerophilus* was not tested by this group. In the here presented study, cross-reactions against the somatic and E/S antigens were detected in sera of dogs infected with *C. vulpis*, *D. immitis, D. repens* and *E. aerophilus.* In contrast, the sandwich-ELISAs using adult somatic antigen purified with mAb were highly specific without a reduction of sensitivity. *C. vulpis* and *E. aerophilus* are distributed over Europe, *D. immitis* is actually still limited to Southern Europe, while *D. repens* has been recently shown to be spreading from eastern and southern to central Europe (Genchi et al., 2011). All these nematode species represent therefore a diagnostic challenge for *A. vasorum* serology. In particular, *A vasorum* and *D. immitis* share

common epitopes and induce strong cross-reactions, as confirmed by cross-reactions using adult somatic antigen in the direct ELISA in the here presented study. Consequently, the produced mAbs were evaluated with E/S antigen of both, adult *A. vasorum* and *D. immitis.* Several mAbs strongly reacting with *D. immitis* were excluded, leading to a highly specific sandwich-ELISA. This is confirmed by the ELISAs with crude antigens, identifying the abovementioned cross-reactions with *D. immitis* and also with *D. repens*. Further dogs with helminth infections such as *A. caninum* and *Echinococcus multilocularis* were tested for cross-reactions because these parasites accomplish a comparable migration within their host, however none were detected.

Sensitivities of all tests were very high in the experimentally infected dogs, while for naturally infected dogs the L1 somatic antigen showed a low sensitivity (42.9%, CI 21.8-66.0%) compared to the E/S antigen or the ELISA using mAb-based purified *A. vasorum* adult somatic antigen. However, more field studies have to be performed and critically analysed to determine the predictive values of different test combinations including specific antibody detection and specific detection of circulating antigens (Schnyder et al, 2011) in different endemic areas.

Specific antibodies were regularly detected between 13 and 21 dpi, depending on the antigens used. Earliest antibody-responses were obtained using L1 antigen, followed by the adult E/S antigen. Also, tests performed using mAb-based purified antigens detected antibodies between 35 and 48 dpi, thus before or around beginning of patency. In dogs treated at 4 dpi, poor antibody-reactions were observed, indicating that both mAbs used might be directed against epitopes of pre-adult and adult stages.

The serological follow-up after anthelmintic treatment of *A. vasorum*-infected dogs showed a continuous antibody decrease after drug administration. Success of anthelmintic treatment

was confirmed through negative faecal analysis. The experimental set-up with *A. vasorum* infected dogs delivered indications concerning the time necessary after treatment (2-9 weeks) for a negative serological antibody-result. The ELISA may be therefore used as a control test for efficacy of anthelmintic treatments against *A. vasorum*. In dogs treated at 4 dpi, virtually no antibody-reaction was observed, with the exception of the ELISA performed with L1 antigen of *A. vasorum*. A possible explanation for this exception would be the presence of L4 at that time point of infection, sharing common epitopes (Guilhon and Cens, 1973).

Concerning the decision which of the crude antigens could be routinely employed, it has to be taken into consideration that the production of *A. vasorum* adult E/S antigen is not only time-consuming but also requires living worms for cultivation. This purpose can be attained only by freshly euthanized infected dogs or foxes and through a delicate procedure as previously described (Schnyder et al., 2009; Schnyder et al., 2010a). In contrast, the adult somatic antigen which was employed in the sandwich-ELISAs is easier to produce since worms can be obtained from sections of hunted foxes. An alternative antigen would be the L1 antigen which unfortunately provides a low sensitivity. Jefferies et al. (2011b) identified immune-reactive adult *A. vasorum* proteins by 2D electrophoresis and mass spectrometry. Such recombinant proteins could be used in ELISA overcoming the limitations of native antigen preparation. The use of specific mAbs for the purification of *A. vasorum* antigens as here described increased the specificity of the ELISA, in particular when testing dogs with one or more clinical signs compatible with angiostrongylosis (96.2% with mAb Av 5/5 and 94.2% with mAb Av 70/1, instead of 86.5% using adult E/S or adult somatic antigen), but differences were not significant. The mAbs demonstrated their potential for further development of highly specific diagnostic tests. Similarly, in an earlier performed study with the related species *A. cantonensis*, a L5 antigen purified with a mAb able to recognize a specific epitope was used in an ELISA, resulting in high sensitivity and specificity (Chye et

al., 2000). Different reasons may explain the positive antibody reaction in sera from dogs with symptoms compatible with angiostrongylosis but negative by the Baermann technique. Faecal samples were correctly sampled over three days to account for intermittent larval shedding. However, larval migration techniques still may miss some *A. vasorum* infected dogs as previously shown (Verzberger-Epshtein et al., 2008). In addition, dogs in the prepatent period or dogs treated with anthelmintics possibly sterilizing adult female worms would result negative in Baermann-analysis. Finally, it has also to be considered that cross-reactions in ELISA may occur. In order to clarify these contradicting results, sera could be further analysed through species-specific antigen (Schnyder et al., 2011) or DNA detection (Jefferies et al., 2009), or through an additional faecal sample a few weeks later if clinical signs are persisting.

The sensitivity of larval detection in faecal samples can be increased by multiple faecal examinations, as reported from recent prevalence studies (Barutzki and Schaper, 2009; Taubert et al., 2008). Diagnostic testing with ELISA represents significant advantages: it enables to diagnose infection with *A. vasorum* before patency, requiring a single serum sample instead of repeated faecal samples and it has the potential for a rapid diagnostic test. The serological detection of antibodies through ELISA is actually a valid and affordable method for diagnoses both in individuals and in population studies. The latter approach may increase the knowledge concerning the epidemiological situation and therefore disease awareness of this potentially fatal parasite for dogs. In fact, due to low compliance of animal owners for multiple collections of faeces and difficulties to always assure a proper storage of faecal samples, *A. vasorum* often remains undetected until dogs are affected by severe respiratory distress or coagulopathies. It has also to be considered that anthelmintic treatments of dogs performed 4-12 x per year (depending on the risk group) as i.e. recommended by the European Counsel for Companion Animal Parasites (http://www.ESCCAP.org) are not

necessarily eliminating metastrongyles such as *A. vasorum*. For an effective treatment, representatives of the group of the macrocyclic lactones such as moxidectin (single topic application, Schnyder et al., 2009) or milbemycin-oxime (weekly oral administration over 4 weeks, Conboy, 2004) are increasingly substituting compounds such as fenbendazole, which is recommended to be administered orally over 20 days (Willesen et al., 2007). The early detection of infections with *A. vasorum* allows a prompt anthelmintic intervention avoiding the onset of severe pathological changes which are possible even in the absence of clinical signs.

Acknowledgement

The authors thank Dr. med. vet. Barbara Riond from the Clinical Laboratory for the sera of blood donor dogs as well as of further randomly selected samples. This work represents the dissertation of Angela Cristina Schucan, veterinarian.

References

Al-Sabi, M.N., Deplazes, P., Webster, P., Willesen, J.L., Davidson, R.K., Kapel, C.M., 2010. PCR detection of *Angiostrongylus vasorum* in faecal samples of dogs and foxes. Parasitol. Res. 107, 135-140.

Barutzki, D., Schaper, R., 2009. Natural infections of *Angiostrongylus vasorum* and *Crenosoma vulpis* in dogs in Germany (2007-2009). Parasitol. Res. 105 Suppl 1, S39-48.

Clopper, C.J., Pearson, E.S., 1934. The use of confidence or fiducial limits illustrated in the case of the binomial. Biometrika 26, 404-413.

383 Conboy, G., 2004. Natural infections of *Crenosoma vulpis* and *Angiostrongylus vasorum* in
384 dogs in Atlantic Canada and their treatment with milbemycin oxime. Vet. Rec. 155,
385 16-18.

386 Chye, S.M., Chang, J.H., Yen, C.M., 2000. Immunodiagnosis of human eosinophilic
387 meningitis using an antigen of *Angiostrongylus cantonensis* L5 with molecular weight
388 204 kD. Acta Trop. 75, 9-17.

389 Cury, M.C., Guimaraes, M.P., Lima, W.S., Vitor, R.W., 2002. Western blot analysis of the
390 humoral response of dogs experimentally infected with *Angiostrongylus vasorum*
391 (Baillet, 1866). Vet. Parasitol. 106, 83-87.

392 Cury, M.C., Lima, W.S., Vitor, R.W.A., 1996. Enzyme-Linked Immunosorbent Assay
393 (ELISA) for the diagnosis of *Angiostrongylus vasorum* (Baillet, 1866) infection in
394 dogs. Revue Méd. Vét. 147, 525-530.

395 De Oliveira Vasconcelos, V., Wagner De Almeida, V.R., Dos Santos Lima, W., 2008.
396 Identification of stage-specific proteins of *Angiostrongylus vasorum* (Baillet, 1866)
397 Kamensky. Parasitol. Res. 102, 389-395.

398 de StGroth, S.F., Scheidegger, D., 1980. Production of monoclonal antibodies: strategy and
399 tactics. J. Immunol. Methods 35, 1-21.

400 Deplazes, P., Smith, N.C., Arnold, P., Lutz, H., Eckert, J., 1995. Specific IgG1 and IgG2
401 antibody responses of dogs to *Leishmania infantum* and other parasites. Parasite
402 Immunol. 17, 451-458.

403 Eckert, J., Friedhoff, K.T., Zahner, H., Deplazes, P. 2008. Lehrbuch der Parasitologie für die
404 Tiermedizin (Stuttgart, Enke Verlag).

405 Fahrion, A.S., Staebler, S., Deplazes, P., 2008. Patent *Toxocara canis* infections in previously
406 exposed and in helminth-free dogs after infection with low numbers of embryonated
407 eggs. Vet. Parasitol. 152, 108-115.

408 Ferdushy, T., Kapel, C.M., Webster, P., Al-Sabi, M.N., Gronvold, J., 2009. The occurrence of
409 Angiostrongylus vasorum in terrestrial slugs from forests and parks in the Copenhagen
410 area, Denmark. J. Helminthol. 83, 379-383.

411 Genchi, C., Kramer, L.H., Rivasi, F., 2011. Dirofilarial infections in Europe. Vector Borne
412 Zoon. Dis, DOI: 10.1089/vbz.2010.0247.

413 Guilhon, J., Cens, B., 1973. *Angiostrongylus vasorum* (Baillet, 1866): Étude biologique et
414 morphologique. Ann. Parasitol. Hum. Comp. 48, 567-596.

415 Jefferies, R., Morgan, E.R., Shaw, S.E., 2009. A SYBR green real-time PCR assay for the
416 detection of the nematode *Angiostrongylus vasorum* in definitive and intermediate
417 hosts. Vet. Parasitol. 166, 112-118.

418 Jefferies, R., Morgan, E.R., Helm, J., Robinson, M., Shaw, S.E., 2011a. Improved detection
419 of canine *Angiostrongylus vasorum* infection using real-time PCR and indirect ELISA.
420 Parasitol. Res. doi 10.10007/s00436-011-2414-4.

421 Jefferies, R., Morgan, E.R., Shaw, S., Heesom, K., 2011b. Identification of immuno-reactive
422 adult *Angiostrongylus vasorum* proteins using mass spectrometry. Mol. Biochem.
423 Parasitol. 180, 56-61.

424 McGarry, J.W., Morgan, E.R., 2009. Identification of first-stage larvae of metastrongyles
425 from dogs. Vet. Rec. 165, 258-261.

426 Oliveira-Junior, S.D., Barcante, J.M., Barcante, T.A., Dias, S.R., Lima, W.S., 2006. Larval
427 output of infected and re-infected dogs with *Angiostrongylus vasorum* (Baillet, 1866)
428 Kamensky, 1905. Vet. Parasitol. 141, 101-106.

429 Schnyder, M., Fahrion, A., Ossent, P., Kohler, L., Webster, P., Heine, J., Deplazes, P., 2009.
430 Larvicidal effect of imidacloprid/moxidectin spot-on solution in dogs experimentally
431 inoculated with *Angiostrongylus vasorum*. Vet. Parasitol. 166, 326-332.

432 Schnyder, M., Fahrion, A., Riond, B., Ossent, P., Webster, P., Kranjc, A., Glaus, T.,
433 Deplazes, P., 2010a. Clinical, laboratory and pathological findings in dogs
434 experimentally infected with *Angiostrongylus vasorum*. Parasitol. Res. 107, 1471-
435 1480.

436 Schnyder, M., Maurelli, M.P., Morgoglione, M.E., Kohler, L., Deplazes, P., Torgerson, P.,
437 Cringoli, G., Rinaldi, L., 2010b. Comparison of faecal techniques including FLOTAC
438 for copromicroscopic detection of first stage larvae of *Angiostrongylus vasorum*.
439 Parasitol. Res. 109, 63-69.

440 Schnyder, M., Tanner, I., Webster, P., Barutzki, D., Deplazes, P., 2011. An ELISA for
441 sensitive and specific detection of circulating antigen of *Angiostrongylus vasorum* in
442 serum samples of naturally and experimentally infected dogs. Vet. Parasitol. 179, 152–
443 158.

444 Staebler, S., Grimm, F., Glaus, T., Kapel, C.M., Haller, M., Hasler, A., Hanosset, R.,
445 Deplazes, P., 2006. Serological diagnosis of canine alveolar echinococcosis. Vet.
446 Parasitol. 141, 243-250.

447 Taubert, A., Pantchev, N., Vrhovec, M.G., Bauer, C., Hermosilla, C., 2008. Lungworm
448 infections (*Angiostrongylus vasorum, Crenosoma vulpis, Aelurostrongylus abstrusus*)
449 in dogs and cats in Germany and Denmark in 2003-2007. Vet. Parasitol. 159, 175-180.

450 Traversa D., Aste G., Milillo G., Capelli G., Pampurini F., Tunesi C., Santori D., Paoletti B.,
451 Boari A., 2010. Autochthonous foci of canine and feline infections by *Dirofilaria*
452 *immitis* and *Dirofilaria repens* in central Italy. Vet. Parasitol. 169, 128-132.

453 Traversa D., Latrofa M. S., Di Cesare A., Meloni S., Castagna S., Otranto D., 2011.
454 Phylogenesis of *Capillaria aerophila* (Trichocephalida, Trichuridae) from different
455 hosts and countries. Proceedings of the 23rd Conference of the WAAVP, 21-24
456 August 2011, Buenos Aires, Argentina.

457 Verzberger-Epshtein, I., Markham, R.J., Sheppard, J.A., Stryhn, H., Whitney, H., Conboy,
458 G.A., 2008. Serologic detection of *Angiostrongylus vasorum* infection in dogs. Vet.
459 Parasitol. 151, 53-60.

460 Willesen, J.L., Kristensen, A.T., Jensen, A.L., Heine, J., Koch, J., 2007. Efficacy and safety of
461 imidacloprid/moxidectin spot-on solution and fenbendazole in the treatment of dogs
462 naturally infected with *Angiostrongylus vasorum* (Baillet, 1866). Vet. Parasitol. 147,
463 258-264.

464

465 Table 1: Evaluation of *Angiostrongylus vasorum* antigens (somatic, excretory/secretory (E/S)
466 or purified by monoclonal antibodies [mAb])) in ELISAs for the specific antibody detection
467 in defined canine sera (CI confidence interval).

	Sensitivity % (95% CI)		Specificity % (95% CI)	
A. vasorum antigens	Natural *A. vasorum* infections[3] (n=21)	Experimental *A. vasorum* infections[3] (n=44[4])	Random samples[5] (n=82)	Cardio-pulmonary diseases[6] (n=52)
Adult E/S[1]	85.7 (63.7-97.0)	100 (93.4-100)	98.8 (93.4-100)	86.5 (74.2-94.4)
Adult somatic[2]	76.2 (52.8-91.8)	97.7 (88.0-99.9)	98.8 (93.4-100)	86.5 (74.2-94.4)
L1 somatic[2]	42.9 (21.8-66.0)	100 (93.4-100)	98.8 (93.4-100)	82.7 (69.7-91.8)
Adult somatic purified with mAb Av 70/1[2]	81.0 (58.1-94.6)	100 (93.4-100)	98.8 (93.4-100)	94.2 (84.1-98.8)
Adult somatic purified with mAb Av 5/5[2]	81.0 (58.1-94.6)	100 (93.4-100)	98.8 (93.4-100)	96.2 (86.8-99.5)

468 [1, 2] Cut-off determination: Mean value of optical density (A_{405nm}) plus 4^1 or 3^2 standard
469 deviations (SD) of thirty healthy blood donor dogs. Four SD were applied where a better

discrimination between positive and negative sera was obtained, based on the results with predefined sera.

[3] Patent infections confirmed by the presence of L1 of *A. vasorum* isolated from faeces by the Baermann-Wetzel technique

[4] Samples of six experimentally infected dogs during patency

[5] Sera of randomly selected dogs without suspicion of a parasitological disease tested at the Clinical Laboratory of the Vetsuisse Faculty of the University of Zurich

[6] Sera of dogs with one or more clinical signs compatible with angiostrongylosis. Larval detection was negative in pooled faecal samples collected over 3 days and analysed with the Baermann-Wetzel technique (Barutzki and Schaper, 2009)

Table 2: Evaluation of *Angiostrongylus vasorum* antigens (excretory/secretory [E/S], somatic or purified by monoclonal antibodies [mAb]) for potential cross-reactions in ELISA with sera of dogs with defined helminth infections.

	No. of sera with cross-reactions					
Angiostrongylus vasorum antigens	*Ancylostoma caninum*[3] (n=4)	*Crenosoma vulpis*[4] (n=9)	*Dirofilaria immitis*[5] (n=20)	*Dirofilaria repens*[6] (n=5)	*Eucoleus aerophilus*[7] (n=6)	Alveolar echino-coccosis[8] (n=8)
Adult E/S[1]	0	2	1	2	1	0
Adult somatic[2]	0	2	4	5	3	0
L1 somatic[2]	0	2	2	4	1	0
Adult somatic purified with mAb Av 70/1[2]	0	1	0	0	0	0
Adult somatic purified with mAb Av 5/5[2]	0	0	1	0	0	0

[1] [2] Cut-off determination as defined in Table 1

[3] Sera of experimentally infected dogs (kindly provided from the Institute for Parasitology, University of Veterinary Medicine, Hannover)

[4] Patent infections confirmed by the presence of L1 of *C. vulpis* in faeces isolated by the Baermann-Wetzel technique

489 [5] Positive for antigen (Diro-CHECK®) or microfilariae of *D. immitis* (characterized with acid
490 phosphatase stain)

491 [6] Naturally infected dogs diagnosed through isolation of microfilariae by Knott-test and
492 confirmed by genetic analyses of part of the mitochondrial *cox*1 gene.

493 [7] Naturally infected dogs diagnosed by flotation procedures of faecal samples with sugar
494 solution, (specific gravity (s.g.) 1.200), and zinc sulphate solution (s.g. 1.350) and confirmed
495 by PCR-coupled sequencing of part of the mitochondrial *cox*1 gene.

496 [8] Naturally infected dogs confirmed by PCR or histology of liver lesions (Staebler et al.,
497 2006).

498

Figure captions

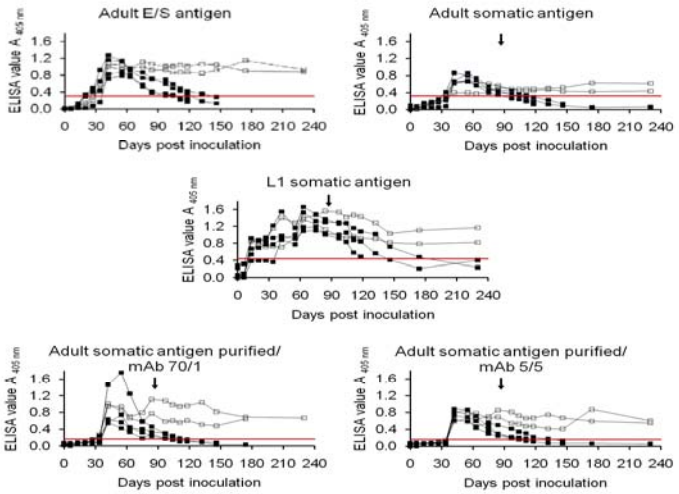
Figure 1:

Specific detection of serum antibodies (IgG) in ELISA against *Angiostrongylus vasorum* adult excretory/secretory (E/S), *A. vasorum* adult somatic and *A. vasorum* L1 somatic antigen, and two adult somatic antigens purified by monoclonal antibodies mAb Av 70/1 or mAb Av 5/5) in six dogs experimentally infected with *A. vasorum.* Four dogs were treated with imidacloprid/moxidectin spot-on (Advocate®) 88dpi (solid symbols) and two dogs were left untreated (open symbols). The time of treatment is marked with an arrow. The cut-off-value (horizontal line) was calculated as mean plus 4 (for ELISA with adult E/S antigen) or 3 (for the other ELISAs) standard deviations (SD) of the ELISA A_{405nm} values of sera from 30 healthy blood donor dogs.

Figure 2:

Specific detection of serum antibodies (IgG) in ELISA against *Angiostrongylus vasorum* adult excretory/secretory (E/S), *A. vasorum* adult somatic and *A. vasorum* L1 somatic antigen, and two adult somatic antigens purified by monoclonal antibodies mAb Av 70/1 or mAb Av 5/5) in ten dogs experimentally infected with *A. vasorum.* Five dogs were treated 32 dpi (left column) and five dogs were treated 4 dpi (right column) with imidacloprid/moxidectin spot-on (Advocate®). The time of treatment is marked with an arrow. The horizontal line represents the cut-off-value, (for determination see Fig. 1).

520 Fig. 1



521

522

523

524

525

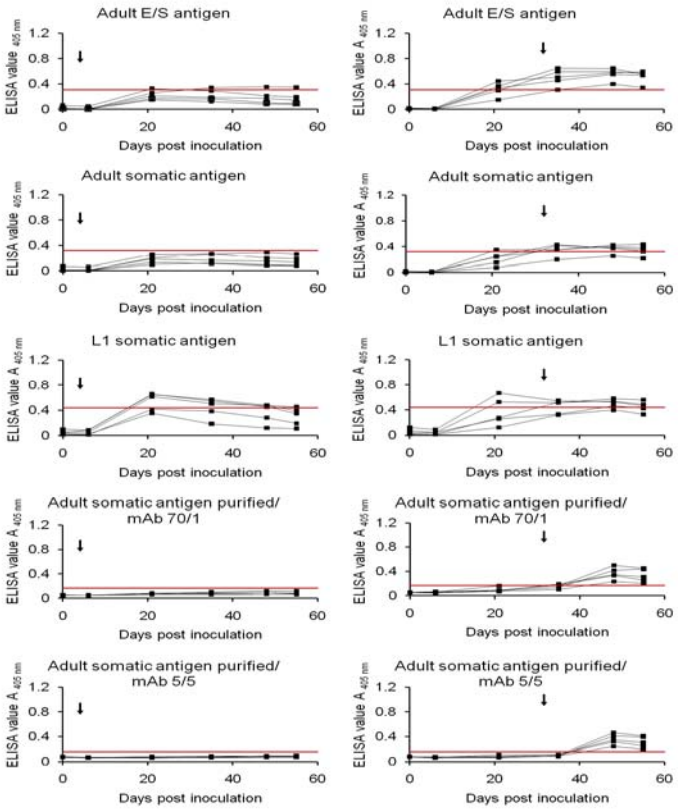
526

527

528

529

530 Fig. 2


Adult E/S antigen
Adult E/S antigen
Adult somatic antigen
Adult somatic antigen
L1 somatic antigen
L1 somatic antigen
Adult somatic antigen purified/ mAb 70/1
Adult somatic antigen purified/ mAb 70/1
Adult somatic antigen purified/ mAb 5/5
Adult somatic antigen purified/ mAb 5/5
ELISA value A 405 nm
Days post inoculation
0
0.4
0.8
1.2
0
20
40
60

531